# Detection of mosaicism at blastocyst stage with the use of high-resolution next-generation sequencing

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A significant proportion of human preimplantation embryos produced during the course of in vitro fertilization (IVF) treatments contain two or more cytogenetically distinct cell lines. This phenomenon, known as chromosomal mosaicism, can involve the presence of cells with different types of aneuploidy in the absence of any normal cells or a mixture of euploid and abnormal cells. Although a high prevalence of mosaicism at the cleavage and blastocyst stages has been appreciated for two decades, the precise frequency of the phenomenon and its consequences for embryo viability have been difficult to quantify. Recent advances in genetic technologies, such as high-resolution next-generation sequencing, have allowed mosaicism to be detected with much greater sensitivity than earlier methods. The application of these techniques to trophectoderm biopsies, taken from embryos before transfer to the uterus, has provided insight into the clinical impact of mosaicism. Data from recent studies show that blastocysts associated with mosaic trophectoderm biopsy specimens implant less often than embryos with a chromosomally normal biopsy. In addition, the mosaic embryos that succeed in establishing a pregnancy are at a significantly higher risk of miscarriage. Because mosaic embryos are less likely to produce a viable pregnancy than their euploid counterparts, we suggest that they are given a lower priority for transfer to the uterus. However, because these embryos can sometimes produce successful pregnancies, it is important that they can be considered for transfer in the absence of fully euploid embryos and after appropriate patient counseling. Unlike aneuploidy of meiotic origin, mosaicism, which is caused by mitotic errors occurring after fertilization, does not increase with advancing maternal age. There may, however, be clinical, treatment, or patient-related factors that contribute to the risk of mosaicism occurring. This review discusses the validation of methods that permit the detection of chromosomal mosaicism in IVF embryos and findings of clinical relevance. (Fertil Steril® 2017;107:1085–91. ©2017 The Authors. Published by Elsevier Inc. on behalf of the American Society for Reproductive Medicine. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).)

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## DETECTION OF MOSAIC BLASTOCYSTS AND THEIR FREQUENCY

Mosaic preimplantation embryos contain two or more cell lines with a different chromosome content, the consequence of errors in chromosome segregation occurring during mitotic divisions. Most studies involving the analysis of mosaic embryos have been performed with the use of fluorescence in situ hybridization (FISH), a method favored because it provides information on the cytogenetic status of each cell. However, the frequency of embryonic mosaicism reported in the literature after FISH varies greatly, ranging from ~30% (1–7) to as high as 90% (8, 9). There are at least four reasons for these differences. One is technical, because FISH requires cell fixation, a technique that is difficult to master and with various alternative protocols available, some of which are associated with significantly higher error rates than others (10). Another, as recently reviewed by Capalbo et al. (11), is the criteria used to classify an embryo as abnormal. Some studies considered an embryo to be mosaic if just one of eight cells appeared to be cytogenetically distinct, whereas others used criteria that were more stringent, and arguably more appropriate, in which an embryo was considered to be mosaic only if it contained several cells with identical abnormalities (e.g., chromosome losses due to anaphase lag), reciprocal aneuploidy (monosomic and trisomic cell lines involving the same chromosome), or polyploidy (which can not be caused by fixation artifacts). The third reason is bias introduced by the type of material tested. Many studies focused on poor-quality material, including arrested embryos, which are more often mosaic

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than their counterparts of good morphology (3, 6). The fourth explanation for differences in reported mosaicism rates is that mosaicism can be iatrogenic—influenced by culture conditions (temperature, pH, media composition, etc.)—and therefore varies from clinic to clinic (12). The combination of unsuitable fixation techniques, insufficiently stringent criteria for defining mosaicism, and sample populations composed largely of arrested embryos, yields apparent mosaicism rates of 90%, but this is not representative of the biologic reality for most embryos. Studies using appropriate FISH methods provided consistent frequencies of mosaicism, with ~30% of embryos at the cleavage stage affected (4–7) and similar rates observed in blastocysts (5, 13).

Molecular cytogenetic techniques (e.g., array comparative genome hybridization [aCGH], single-nucleotide polymorphism [SNP] array, quantitative polymerase chain reaction [qPCR], next-generation sequencing [NGS]) have the advantage over FISH that they can provide information on the copy number of all 24 types of chromosome. In contrast, FISH studies typically examined only about one-third to one-half of the chromosomes in each cell. Unfortunately, these methods become relatively expensive when many individual cells need to be assessed, and consequently they have rarely been applied to disaggregated embryos as would be required for a definitive study of mosaicism (14, 15). Most research using comprehensive chromosome screening technologies have involved the analysis of blastocyst biopsy specimens, typically composed of ~5 cells, which are not separated but instead are analyzed as a single entity. Although the presence of a mixture of normal and aneuploid cells in the specimen can sometimes be detected with the use of methods such as aCGH, qPCR, and SNP array, they are relatively insensitive for this purpose. If ideal results are obtained, mosaicism associated with proportions of aneuploid cells ranging from 40% to 60% can be detected with a high degree of confidence. However, proportions of abnormal cells outside this range will frequently be indistinguishable from either normality (when there are few abnormal cells) or nonmosaic aneuploidy (when the majority of cells in the sample are aneuploid).

The method with the greatest power to detect mosaic samples is the relatively new technique of high-resolution next-generation sequencing (hr-NGS). Using hr-NGS, one study suggested that 21% of blastocyst biopsy samples contain a mixture of euploid and abnormal cells, and that a further 10% are mosaic for two or more different aneuploid lines. Those embryos found to be mosaic with the use of hr-NGS had proportions of aneuploid cells ranging from 20% to 80% (Liu et al., unpublished data). These results are similar to those of historical FISH studies, which analyzed all cells individually. In contrast to the findings from hr-NGS, a recent study using aCGH reported a mosaicism rate of only 4.8% in blastocyst biopsy specimens, with the proportion of aneuploid cells ranging from 35% to 50% (16). The higher rate of mosaicism detected by hr-NGS is likely explained by a superior sensitivity of this method for detecting minor lines in mixed cell populations compared with aCGH.

Interestingly, unlike aneuploidy of meiotic origin, the incidence of mosaic chromosomal abnormality does not change with advancing years, with ~30% of blastocyst-stage embryos affected across all maternal ages. However, because meiotic errors are more common in the embryos of older mothers, the percentage of blastocysts with biopsy specimens containing only euploid cells declines as a woman ages, falling from 48.2% for women <35 years of age to 10.6% of blastocysts for patients >42 years of age. Over the same period of time, the proportion of embryos with mosaic biopsies that include a normal cell line falls from 26.6% to 10.5%. The mitotic errors, leading to mosaicism, coupled with the advancing risk of meiotic aneuploidy has clinical implications for in vitro fertilization (IVF) treatments, especially those using preimplantation genetic screening for aneuploidy (PGS-A), because it effects the likelihood of detecting an entirely euploid embryo for transfer.

It is important to note that not all NGS strategies deliver the same information. Depending on the depth of sequencing and the specific NGS platform used, the sensitivity for detecting cytogenetically distinct subpopulations of cells varies. Considering that most blastocyst biopsies contain ~5 cells, the ability to detect of <20% abnormal cells (i.e., less than one abnormal cell out of five) or >80% aneuploidy (more than four abnormal cells out of five) is probably not relevant in the context of PGS-A. Nonetheless, it is important that aneuploidy in the 20%–80% range is consistently and reliably detected, because mosaicism in this range has clinical implications (discussed in detail below). Although some NGS methods have been validated for mosaicism detection (17, 18), questions remain as to the ability of other techniques to reliably detect this phenomenon, e.g., copy number variation sequencing (19), EmbryVu, qPCR, and other lower-resolution methods used for PGS-A.

## VALIDATION OF MOSAICISM DETECTION WITH THE USE OF HR-NGS

The most widely used highours resolution NGS method is the VeriSeq PGS system (Illumina). This involves sequencing on a benchtop device called a MiSeq, which yields ~24 million short fragments of DNA sequence, known as "reads," per run. Not all of these sequences are necessary for enumeration of chromosome copy number, and, to make the test cheaper, it is usual for several DNA samples to be "barcoded" and analyzed simultaneously during the same run. In general, 60%–70% of reads can be mapped to unique parts of the genome and are therefore suitable for assessing the quantity of DNA from individual chromosomes. Therefore a typical experiment, in which 24 samples are analyzed in parallel, usually provides 600,000–900,000 reads per sample. This is sufficient for the detection of mosaic abnormalities present in 20%–80% of the cells comprising the biopsy sample. The software (BlueFuse Multi v3; Illumina) provides copy number counts for each chromosome pair. A chromosome with two copies is considered to be euploid, a chromosome with one copy monosomic, and a chromosome with three copies trisomic. Values that fall between the thresholds used for assigning one, two, or three chromosome copies may be